BETA-GLUCOSIDASE OF THE MIDGUT OF THE SILKWORM BOMBYX MORI

TOSHIO ITO AND MOTOZÔ TANAKA

Sericultural Experiment Station, Suginami-ku, Tokyo, Japan

Contrary to a wide distribution of β-glucosidase in plants, the occurrence of this enzyme in insects seems to be rare, since neither utilization of β-glucosides nor presence of the enzyme activity has been often recognized in insects. Until recently, the demonstration of this enzyme in insects has been discussed on the basis of the utilization of β-glucosides in growth experiments (see the review by Lipke and Fraenkel, 1956). Studies of this enzyme from the enzymic points of view, however, have been lately carried out with the wood louse *Porcellio* (Newcomer, 1952, 1956) and the cockroach *Periplaneta americana* (Newcomer, 1954). Applying a highly sensitive fluorimetric method for β-glucosidase assay, Robinson (1956) has demonstrated the occurrence of the enzyme in the locust *Locusta migratoria*, the mealworm *Tenebrio molitor*, the water-boatman *Notonecta*, the cockroach *Periplaneta americana*, and the black aphis *Aphis fabae*. The occurrence of this enzyme has also been reported for the bean weevil *Callosobruchus chinensis*, the bean blister beetle *Epicauta gorhami*, the silkworm *Bombyx mori*, and the wild silkworm *Dictyoploca japonica* (Koike, 1954), and for the mealworm *Tenebrio molitor* (Fraenkel, 1955).

A few years ago the present authors became aware of the fact that the midgut homogenate of the silkworm is able to hydrolyze salicin, but the digestive fluid gave scarcely the same reaction. Recently, this problem was re-investigated to obtain more detailed results. This report is mainly concerned with the occurrence of β-glucosidase in the silkworm midgut, its characterization, and partial purification. A comparison of the enzyme activity of the normal larvae was also made with amylase-free mutants, and with jaundice-diseased larvae.

Materials and Methods

Practical methods of obtaining midgut homogenates have been previously reported (Ito, Horie and Ishikawa, in press; Ito and Horie, in press). Homogenates made in water were used directly in some experiments, but the acetone powder made with midgut homogenates was used for most enzyme preparations. Midgut homogenates made from middle fifth instar larvae were dehydrated by mixing with 7 volumes of chilled acetone and the precipitates were collected in a Büchner funnel under suction. The precipitates were subsequently re-suspended in chilled acetone, then separated from acetone with funnel as above. The precipitates were washed by running alcohol-ether mixture (1:1) and brought to dryness in a vacuum desiccator. The dried, pale-yellow cake was ground in a mortar and the acetone powder thus made was used for enzyme tests. The powder was kept *in vacuo* at 5° C. at least for 8 months without any loss of β-glucosidase activity. Preparing the enzyme solution, the powder was suspended in water, allowed to stand for two hours at 5° C.,

and the supernate, obtained after centrifugation at 10,000 × g for 10 minutes, was used for the experiments.

Digestive fluid was collected from middle fifth instar larvae by applying a weak electric shock to them. The fluid was either used for enzymic measurements directly after dialysis against water at 5° C. for 48 hours, or after conversion to an acetone powder.

Enzyme activity was assayed by measuring the amount of glucose liberated from β-glucoside in the reaction system. Salicin was used as a substrate in most experiments, and cellobiose or phenyl β-glucoside in some. Unless otherwise indicated, each reaction mixture contained 200 μM citrate buffer (pH 5.4), 48 μM salicin and 1.0 ml. acetone powder solution (total volume 4.0 ml.) and was incubated at 30° C. for two hours. The reaction was stopped at intervals by adding an aliquot to $Ba(OH)_2$ and $ZnSO_4$, or Na_2WO_4. When cellobiose was used as the substrate, the reaction was stopped by Na_2WO_4 and H_2SO_4, and bakers' yeast then applied to the supernate of the reaction mixture in order to remove fermentable sugar. Glucose was determined mainly by the method of Hagedorn and Jensen (1923) and sometimes by Somogyi's procedure (1952).

Nitrogen was determined by the micro-Kjeldahl method.

Results

Optimal pH range

The supernate obtained from an acetone powder suspension was incubated with various buffers at different pH levels. As shown in Figure 1, almost no alteration of pH optimum was found with different buffers. Optimal pH ranges were 5.0–6.2 for citrate and phosphate, and 5.2–6.4 for acetate. These ranges are more extended than those reported for other insects (Newcomer, 1952, 1954, 1956; Robinson, 1956) and for plants (Veibel, 1950). The enzymic activity was relatively high at a high pH level such as 7.0 or even 8.0, which has not been reported so far for other species of insects. The measurement also showed that β-glucosidase activity in borate buffer was not reduced to zero at pH 9.4. It has been known that the pH optimum of this enzyme is dependent on the source of the enzyme and to a minor degree on the substrate and the buffer solution (Veibel, 1950).

Velocity of hydrolysis

The relationship between enzyme concentration and velocity of hydrolysis is shown in Figure 2, where the enzyme concentration was doubled, respectively, from curve 3 to curve 1 (1:2:4). It is apparent that the rate of glucose liberation is proportional to enzyme concentration. Figure 2 also shows that the reaction proceeded at a uniform rate when enzyme concentration was relatively low.

In Figure 3 the effect of the concentration of the substrate on the enzyme activity is shown. The curves were plotted according to the procedure of Lineweaver and Burk (1934), *i.e.*, the inverse of the activity against the inverse of salicin concentration. The *Km* value (the Michaelis constant) is 0.013 M, which is in accord with the value reported for salicin (Veibel and Lillelund, 1938).

Inhibition by high temperature

The effect of high temperature on midgut β-glucosidase is shown in Table I. The supernate obtained from acetone powder suspension was treated at 40 to 70° C.

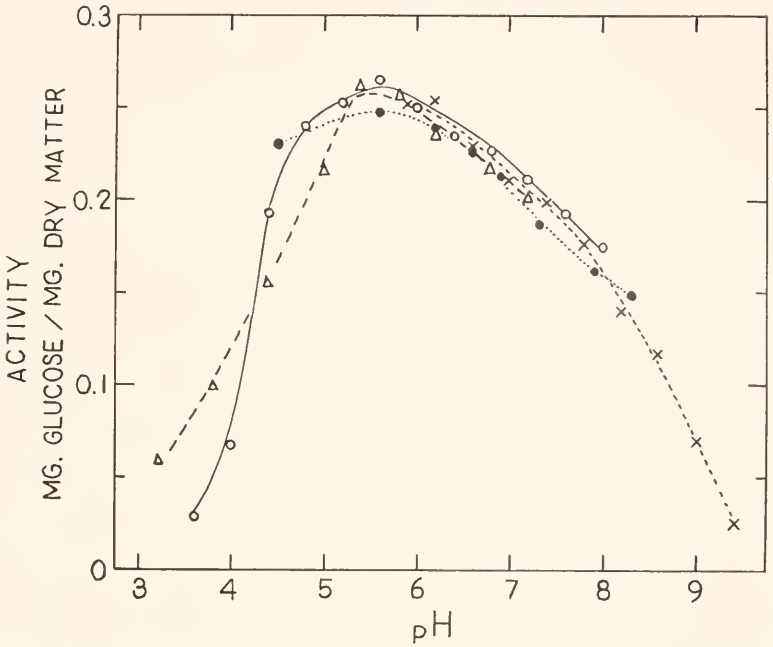
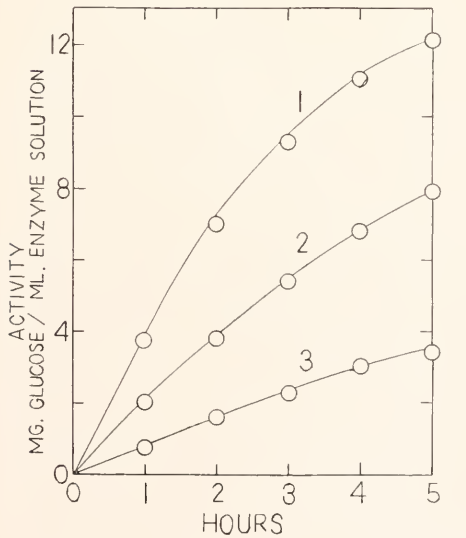


FIGURE 1. Relationship between pH and β-glucosidase activity. Phosphate buffer: ●.....●. Borate buffer: ×--------×. Citrate buffer: ○————○. Acetate buffer: △— — — —△.



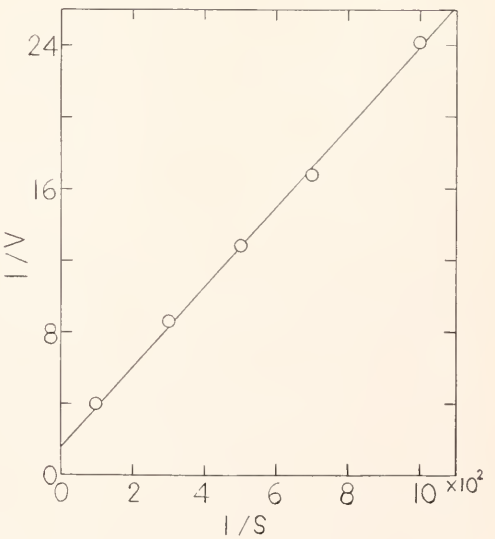


FIGURE 2 (left). β-Glucosidase activity as a function of time for different enzyme concentrations.

FIGURE 3 (right). Relationship between β-glucosidase activity and salicin concentration. Borate buffer (pH 6.0). Total volume, 5.0 ml. Incubation, one hour. Enzyme activity was expressed in terms of glucose liberated per dry matter on the basis of the same weight.

TABLE I

Effect of high temperature on β-glucosidase activity

Time for treatment	Relative activity (%)			
	40° C.	50° C.	60° C.	70° C.
5 min.	102.4	100.3	86.4	9.2
10	100.4	96.1	61.9	4.9
20	94.2	81.6	22.3	4.9
Control	100.0	100.0	100.0	100.0

for 5 to 20 minutes. At 40° C. no effect was observed with a 10-minute exposure and slight inhibition was recognized after exposure for 20 minutes. The treatment at 50° C. for 10 minutes resulted in a slight inhibition and that for 20 minutes in a 20 per cent inhibition. The treatment at 60° C., however, resulted in a markedly increasing loss of the activity according to the prolongation of exposing period up to 80 per cent of inhibition. By applying a high temperature of 70° C., most of the activity was lost within 5 minutes.

Inhibition by heavy metals

In insects the inhibition of β-glucosidase by heavy metals has been reported for the ventriculus of the adult cockroach (Newcomer, 1954). Inhibition of the enzyme solution obtained from the silkworm midgut with varying concentrations of $AgNO_3$ or $HgCl_2$ resulted in varying degrees of inhibition, as shown in Table II.

Effect of organic acids

Inhibition of β-glucosidase by organic acids has been reported for *Penicillium*, when phenyl β-glucoside was used as substrate (Murakami, 1950). Malic, fumaric, and citric acids were tested for their inhibitory effects on silkworm midgut β-glucosidase at a final concentration of 0.05 *M*. The results showed that no appreciable inhibition was observed, when salicin was used as substrate.

Effect of toluene

Newcomer (1954) has shown that an activation of β-glucosidase by toluene does not occur in the cockroach. The effect of toluene on midgut β-glucosidase in the silkworm was tested and no activation was recognized. Toluene was, there-

TABLE II

Inhibition of β-glucosidase activity by heavy metals

Final concentration (*M*)	Inhibition (%)	
	$AgNO_3$	$HgCl_2$
1×10^{-2}	95.3	—
2×10^{-3}	76.4	86.7
1×10^{-3}	57.9	52.8
2×10^{-4}	26.4	23.5
1×10^{-4}	1.1	15.5
0	0.0	0.0

fore, added to the incubation mixture when a long period of incubation was necessary.

Distribution of the activity in the midgut

The activity of β-glucosidase was compared among different parts of the midgut, *i.e.*, anterior, middle, and posterior midguts. The measurement of the activity was carried out with fresh homogenates and the results are shown in Figure 4. It is evident that the majority of the activity is concentrated in the posterior midgut, while a very low activity is found in the anterior and middle midguts.

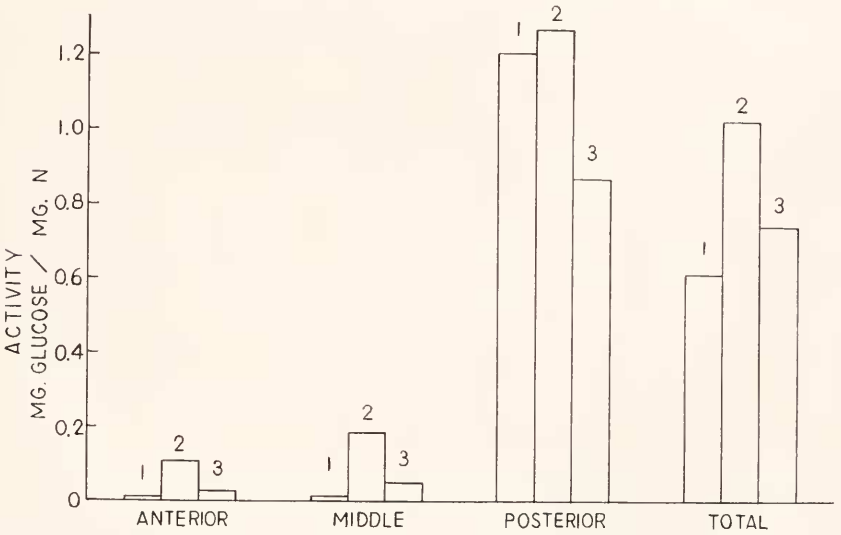


FIGURE 4. Distribution of β-glucosidase activity in the different parts of the midgut. 1, fourth day; 2, seventh day; 3, eighth day of fifth instar.

The change in the activity during larval development

The changes in β-glucosidase activity according to the development were measured with fresh midgut homogenates during fourth and fifth larval instars. The measurements were made with spring silkworms, the rearing temperature ranging approximately from 20 to 25° C., and with summer silkworms, the rearing temperature ranging approximately from 25 to 30° C. Though the activity expressed by unit glucose freed per nitrogen was higher in spring silkworms than in summer silkworms, the changes in the activity were almost the same in both (Fig. 5). In general, the activity was low during the fourth and early fifth instars. A marked increase in the activity occurred at the middle period of the fifth instar, and was maintained for a few days. Then the activity dropped suddenly and reached the lowest level during cocoon-spinning.

Precipitation by ammonium sulfate

In a preliminary experiment it was noticed that the majority of the activity was precipitated between 0.3 and 0.5 saturation with ammonium sulfate, when a suspen-

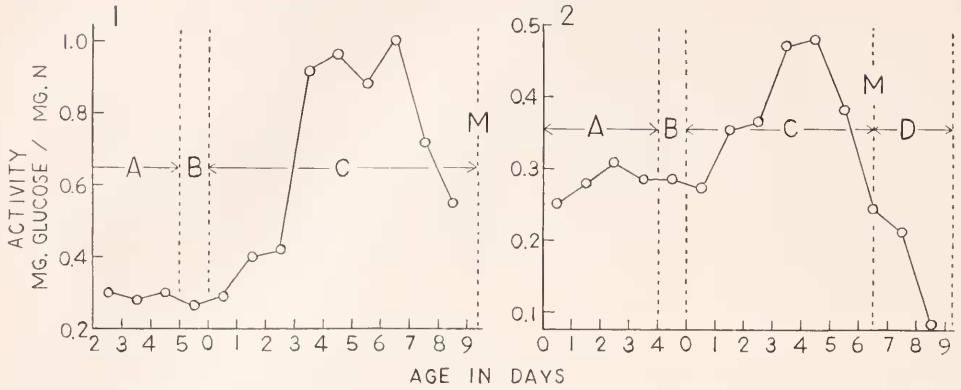


FIGURE 5. Change in β-glucosidase activity during larval development. *1*, spring rearing; *2*, summer rearing. Fresh homogenates were diluted to ½ in spring, and ¼ in summer. A, fourth instar; B, fourth molting period; C, fifth instar; D, cocoon-spinning period; M, maturity.

sion of acetone powder was used. Therefore, an attempt was made to purify β-glucosidase of the midgut by means of ammonium sulfate precipitation. Subsequently, the precipitation procedure was repeated several times by increasing the concentration of ammonium sulfate progressively. Table III shows one of the results obtained. Acetone powder made with posterior midguts was suspended in water in the cold for four hours; this suspension was used for the precipitation experiment. The specific activity of this suspension was 1.83 and that obtained with supernate after centrifugation at 12,000 × *g* for 10 minutes was increased almost three times, as seen in Table III. About 90 per cent of the original activity was found in the supernate. Until 0.350 saturation, very slight activity was precipitated. Most activity was precipitated between 0.350 and 0.450 saturation and the highest specific activity was obtained between 0.375 and 0.425 saturation. The specific activity was increased to about 4 times that of the supernate, and 10 times that of the original suspension. The application of ammonium sulfate precipitation thus seems to be to some extent useful for the purification of β-glucosidase.

TABLE III

Precipitation of β-glucosidase by ammonium sulfate

Saturation of ammonium sulfate	Total activity, mg. glucose	Specific activity, mg. glucose/mg. N	Recovery, %
Suspension	360.67	1.83	100.0
Supernate	327.88	4.37	90.91
0 –0.325	5.23	0.23	1.45
0.325–0.350	8.13	0.84	2.25
0.350–0.375	55.73	9.68	15.45
0.375–0.400	92.00	16.61	25.51
0.400–0.425	75.05	14.92	20.81
0.425–0.450	52.50	7.64	14.55
0.450–0.475	12.23	3.71	3.39
0.475–0.500	7.30	3.97	2.02

Acetone powder used in the present study was recognized to possess amylase and invertase, in addition to β-glucosidase. An attempt was therefore made to separate β-glucosidase from amylase or invertase by means of ammonium sulfate precipitation. The result showed that the precipitates at between 0.375 and 0.425 saturation contained all of three activities at almost the same level (β-glucosidase, 56.5%; amylase, 41.7%; invertase, 57.0%).

Several methods have been presented for the standardization of β-glucosidase (Veibel, 1950). An enzyme efficiency was obtained with a few fractions precipitated by ammonium sulfate by the use of phenyl β-glucoside as the substrate (final concentration, 0.052 *M*), according to the procedure by Helferich (1933, 1938). A high value of enzyme efficiency, 0.898, was obtained with the precipitate at between 0.375 and 0.425 ammonium sulfate saturation, while 0.170 with the precipitate be-

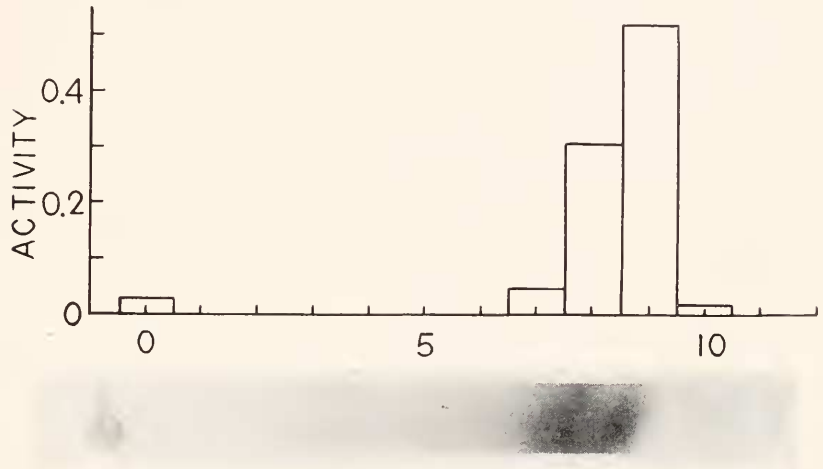


FIGURE 6. Distribution of β-glucosidase activity after paper electrophoresis. The activity was expressed in terms of mg. glucose liberated.

tween 0–0.375 saturation, 0.154 with that between 0.425–0.700 saturation, and 0.095 with the original supernate of acetone powder suspension.

Furthermore, β-glucosidase of the silkworm midgut was recognized to hydrolyze cellobiose as the substrate.

Purification by paper electrophoresis

Robinson (1956) has applied a paper electrophoretic procedure for the separation of β-glucosidase from β-glucuronidase in the locust-crop fluid. A similar procedure was also tested with β-glucosidase from the silkworm midgut. Either an acetone power supernate or the precipitate at 0.425–0.450 saturation of ammonium sulfate was used for the experiment. An enzyme fraction was subjected to electrophoresis on filter paper (Tôyô No. 51) 3 cm. × 30 cm. in 0.1 *M* phosphate buffer at pH 5.8, 150 volts, 5 ma., for 8 hours. Subsequently, the paper was cut into half along the long side of the paper. One of the divided strips was dried and suspended in the staining solution (Amido Black) for proteins, and the other was cut into

one-cm. sections starting from the original spot, each section being chopped into small pieces which were immediately placed into a test tube with 1.0 ml. of phosphate buffer (pH 5.8). The test tube was kept at 5° C. for 5 hours to extract the enzyme, then heated to 37° C. with the addition of an appropriate amount of salicin solution (final concentration, 0.0125 *M*). After a 16-hour incubation, the amount of sugar liberated was determined. The results of the enzymic test, as well as of the staining test on the precipitate by ammonium sulfate, are shown in Figure 6, where the β-glucosidase activity is shown in the form of histograms. β-Glucosidase appeared in the locations corresponding to the staining test on the other strip. Little activity was found at the original point, where a protein band remained. However, when the whole midgut suspension was used, another two protein bands were recognized on the paper, which were considered to have been removed by the procedure of the precipitation with ammonium sulfate. The separation of invertase from β-glucosidase by electrophoresis was unsuccessful.

β-Glucosidase activity in the midgut of jaundice-diseased larvae

Two types of polyhedroses are known to occur in the silkworm, one of which is called cytoplasmic polyhedrosis, with the formation of the polyhedral bodies in the midgut cytoplasm. Several days after the infection, midgut tissue becomes white, which is a typical symptom of this disease. β-Glucosidase activity was compared between normal and infected larvae. The activity was always lower in diseased larvae reduced to 61 per cent of the normal larvae (incubation period, two hours) and 82 per cent (incubation period, 6 hours).

β-Glucosidase activity in the digestive fluid

The activity of the digestive fluid per unit nitrogen of early fourth instar larvae was one-third that of the midgut or less, while that of late fourth instar and fifth instar larvae was less than one-tenth that of the midgut. β-Glucosidase activity was also recognized in the digestive fluid of the amylase-free strain, which is deficient in amylase activity in the digestive fluid. The experiment performed at the same time showed that the midgut of the amylase-free strain possessed the same level of activity of β-glucosidase as the normal strain.

Discussion

The exact physiological role of β-glucosidase of the silkworm midgut in digestion is at present not well understood. The enzyme activity on the basis of the same unit is, however, higher in the midgut than in the digestive fluid. This seems to suggest that β-glucosidase in the midgut cells is of rather more importance than that in the digestive fluid. The optimal pH of midgut β-glucosidase ranges approximately 5.0 to 6.4, while an effort was unsuccessful to determine its range in the digestive fluid. A possible role of β-glucosidase in the cells of the midgut in digestion is also deduced from the fact that the pH value of the digestive fluid is strongly alkaline, as much as 10.0. The movement of food through the gut is generally fast in the silkworm larva, occurring within a few hours. Thus, even though the degree of participation of this enzyme in digestion as a whole is still

unknown, the possibility remains that the mulberry carbohydrates which have not been completely hydrolyzed in the lumen of the gut might be hydrolyzed after absorption in the midgut tissue. It is interesting from the standpoint of comparative physiology that the intercellular enzyme might participate in the digestion. Although conclusions drawn from a study of enzyme alone are generally open to question in regard to the physiological role in intact organs, a good correlation was found between pure compounds supporting growth and the presence of digestive enzymes in insects (Day and Waterhouse, 1953). Koike (1954) could not demonstrate cellulase in the digestive tract of the silkworm and Hiratsuka (1917) has shown that cellulose is not utilized by silkworm larvae. This is the same situation as reported for the hepatopancreas of *Porcellio* (Newcomer, 1956) where an activity of β-glucosidase was demonstrated without that of cellulase. β-Glucosidase of the midgut or of the digestive fluid of the silkworm seems to hydrolyze β-glucosides contained in the mulberry leaves. A few papers have been so far published on glucosidic compounds in the mulberry leaves; recently Hamamura and Naito (1956) isolated arginine β-glucoside and the presence of glucosides of the pigment has also been reported (Oshima and Nakabayashi, 1951). There is no doubt that these glucosides and possibly other not yet identified glucosides are utilized by the larvae.

The results on the characterization experiments suggest that the β-glucosidase of the midgut is very much similar to that in plants (Veibel, 1950). The enzyme efficiency of β-glucosidase of the midgut is rather higher than that obtained with plants (Pigman, 1946).

A variation in the digestive enzyme activities of different parts of the midgut, as well as in the ability of the absorption of the nutrients, is well known in insects (Day and Waterhouse, 1953; Waterhouse and Day, 1953). The physiological or digestive differentiation in the different portions of the midgut of the silkworm is still not well known in many respects. However, the highest activity of β-glucosidase was found in the posterior midgut (Fig. 4). Matsumura and Oka (1935) have shown that the activity of amylase or invertase is also the highest in the posterior midgut. The glycogen content is increased most markedly in the posterior midgut after sugar ingestion (Horie and Tanaka, 1957) and the highest phosphorus metabolism was obtained also in this portion (Ito, Horie and Tanaka, in press).

The authors wish to express their thanks to Prof. G. S. Fraenkel of the University of Illinois for reading the manuscript.

Summary

1. The presence of a β-glucosidase was demonstrated in the midgut of the silkworm larva, *Bombyx mori*.

2. The enzyme has a pH optimum of approximately 5.2–6.2 and the *Km* value was 0.013 with salicin as a substrate.

3. The action of the enzyme was slightly inhibited at a temperature of 40° C., and strongly inhibited at 70° C. An inhibition by silver or mercury salts was also observed, while no inhibition was found by organic acids. No activation by toluene was demonstrated.

4. Most of the activity in the midgut was concentrated in the posterior portion.

5. The enzyme activity varies according to larval growth, being lower at the beginning of the fifth instar, higher after the middle of the instar, and again lower during cocoon-spinning.

6. The enzyme activity was concentrated 10 times by means of ammonium sulfate precipitation at a saturation of 0.375–0.425. Separation by the paper electrophoretic method was successfully applied for this fraction, but it was unsuccessful for separating β-glucosidase from other enzymes.

7. Virus-infected larvae showed a decrease in enzyme activity, compared with normal larvae.

8. β-Glucosidase activity in the digestive fluid was much lower than that in the midgut. A mutant, amylase-free strain possessed in the digestive fluid the same level of β-glucosidase activity as the normal one.

LITERATURE CITED

Day, M. F., and D. F. Waterhouse, 1953. The mechanism of digestion. *In:* Insect Physiology, pp. 311–330. John Wiley & Sons, Inc., New York.

Fraenkel, G., 1955. Inhibitory effects of sugars on the growth of the mealworm, *Tenebrio molitor* L. *J. Cell. Comp. Physiol.*, **45**: 393–408.

Hagedorn, H. C., and B. N. Jensen, 1923. Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. *Biochem. Zeitschr.*, **135**: 46–58.

Hamamura, Y., and K. Naito, 1956. Studies on the micro constituent in mulberry leaves. I. On the isolation of arginine-glucoside from mulberry leaves. *J. Agricul. Chem. Soc. Japan*, **30**: 358–361. (In Japanese with English summary.)

Helferich, B., 1933. Die Spezifität des Emulsins. *Ergebn. Enzymforsch.*, **2**: 74–89.

Helferich, B., 1938. Emulsin. *Ergebn. Enzymforsch.*, **7**: 83–104.

Hiratsuka, E., 1917. Researches on the nutrition of the silk worm. *Bull. Imp. Sericul. Exp. Sta.*, **2**: 353–412. (In Japanese.)

Horie, Y., and M. Tanaka, 1957. Absorption and utilization of glucose in silkworm larvae, *Bombyx mori*. *J. Sericul. Sci. Japan*, **26**: 40–45. (In Japanese with English summary.)

Ito, T., Y. Horie and M. Tanaka. Phosphorus compounds of the midgut in the silkworm. *Proc. 10th Internat. Congr. Entomol.* (in press).

Ito, T., Y. Horie and S. Ishikawa. Oxidative enzymes of the midgut of the silkworm *Bombyx mori*. *J. Insect Physiol.* (in press).

Ito, T., and Y. Horie. Carbohydrate metabolism of the midgut of the silkworm *Bombyx mori*. *Arch. Biochem. Biophys.* (in press).

Koike, H., 1954. Studies on carbohydrases of insects. I. Distribution of carbohydrases in several insects. *Zool. Mag.*, **63**: 228–234. (In Japanese with English summary.)

Lineweaver, H., and D. Burk, 1934. The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.*, **34**: 658–666.

Lipke, H., and G. Fraenkel, 1956. Insect nutrition. *Ann. Rev. Entomol.*, **1**: 17–44.

Matsumura, S., and T. Oka, 1935. Physiological studies on the carbohydrases of the silkworm. *Bull. Nagano-ken Sericul. Exp. Sta.*, **31**: 1–32. (In Japanese.)

Murakami, H., 1950. The effect of organic acids on β-glucosidase of *Penicillium*. *Kagaku* (*Science*), **20**: 326–327. (In Japanese.)

Newcomer, W. S., 1952. The occurrence of beta-glucosidase in the digestive juice of *Porcellio* and *Armadillidium*. *Anat. Rec.*, **113**: 536.

Newcomer, W. S., 1954. The occurrence of β-glucosidase in digestive juice of the cockroach, *Periplaneta americana* L. *J. Cell. Comp. Physiol.*, **43**: 79–86.

Newcomer, W. S., 1956. Digestive carbohydrates of the wood louse, *Porcellio*. *Physiol. Zoöl.*, **29**: 157–162.

Oshima, Y., and T. Nakabayashi, 1951. Studies on tannins and pigments by partition chromatography. I. Analysis of quercetin and its 3-glycosides. *J. Agricul. Chem. Soc. Japan*, **25**: 21–25. (In Japanese with English summary.)

PIGMAN, W. W., 1946. Specificity, classification, and mechanism of action of the glycosidases. *Adv. Enzymol.*, **4**: 41–74.

ROBINSON, D., 1956. The fluorimetric determination of β-glucosidase: its occurrence in the tissues of animals, including insects. *Biochem. J.*, **63**: 39–44.

SOMOGYI, M., 1952. Notes on sugar determination. *J. Biol. Chem.*, **195**: 19–23.

VEIBEL, S., 1950. β-Glucosidase. *In:* The Enzymes, Vol. I, Part 1, pp. 583–620. Academic Press Inc., New York.

VEIBEL, S., AND H. LILLELUND, 1938. Über die Standardisierung von β-glucosidase. *Enzymol.*, **5**: 129–136.

WATERHOUSE, D. F., AND M. F. DAY, 1953. Function of the gut in absorption, excretion, and intermediary metabolism. *In:* Insect Physiology, pp. 331–349. John Wiley & Sons, Inc., New York.